

# Do bednets including piperonyl butoxide offer additional protection against populations of *Anopheles gambiae* s.l. that are highly resistant to pyrethroids? An experimental hut evaluation in Burkina Faso

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**Abstract.** Malaria control is dependent on the use of longlasting insecticidal nets (LLINs) containing pyrethroids. A new generation of LLINs containing both pyrethroids and the synergist piperonyl butoxide (PBO) has been developed in response to increasing pyrethroid resistance in African malaria vectors, but questions remain about the performance of these nets in areas where levels of pyrethroid resistance are very high. This study was conducted in two settings in southwest Burkina Faso, Vallée du Kou 5 and Tengrela, where *Anopheles gambiae* s.l. (Diptera: Culicidae) mortality rates in World Health Organization (WHO) discriminating dose assays were <14% for permethrin and <33% for deltamethrin. When mosquitoes were pre-exposed to PBO in WHO tube assays, mortality rates increased substantially but full susceptibility was not restored. Molecular characterization revealed high levels of *kdr* alleles and elevated levels of P450s previously implicated in pyrethroid resistance. In cone bioassays and experimental huts, PBO LLINs outperformed the pyrethroid-only equivalents from the same manufacturers. Blood feeding rates were 1.6–2.2-fold lower and mortality rates were 1.69–1.78-fold greater in huts with PBO LLINs vs. non-PBO LLINs. This study indicates that PBO LLINs provide greater personal and community-level protection than standard LLINs against highly pyrethroid-resistant mosquito populations.

**Key words.** insecticide resistance, insecticide resistance management, longlasting insecticidal nets, PBO.

## Introduction

Use of the longlasting insecticidal net (LLIN) is pivotal in the fight against malaria in Africa. A massive scaling up of the distribution of this commodity has occurred over the past 15 years, with 178 million LLINs delivered for use in sub-Saharan Africa (SSA) in 2015 alone [World Health Organization (WHO), 2016]. Although reliable estimates of LLIN usage are very hard to obtain, the WHO estimates that 53% of the population at risk

in SSA slept under an LLIN in 2015 (WHO, 2016). The results have been dramatic: an estimated 450 million clinical cases of malaria were averted in the last 15 years by the use of LLINs (Bhatt *et al.*, 2015).

All LLINs in current use contain pyrethroid insecticides, but there is growing recognition that increases in the prevalence and intensity of pyrethroid resistance, driven at least in part by the scale-up in the use of LLINs, could jeopardize recent gains in malaria control (WHO, 2012). Direct evidence that pyrethroid

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resistance is reducing either the personal or community-level protection provided by LLINs is challenging to obtain (Kleinschmidt *et al.*, 2015; Ranson & Lissenden, 2016), but models of malaria transmission predict that even relatively low levels of resistance can substantially reduce the public health benefits of LLINs (Churcher *et al.*, 2016). In countries that rely on the LLIN as the primary malaria prevention tool, the only currently available alternatives to conventional pyrethroid-only LLINs are nets in which the synergist piperonyl butoxide (PBO) has been included in the fibres making up all, or part, of the net. Piperonyl butoxide inhibits cytochrome P450s, which comprise one of the most important enzyme families involved in pyrethroid resistance, and exposure to PBO has been shown to reduce resistance and sometimes to restore susceptibility to pyrethroids in malaria vectors (Jones *et al.*, 2013; Edi *et al.*, 2014).

Four brands of LLIN containing PBO have received interim approval from the WHO as conventional LLINs. These are the PermaNet® 3.0 (deltamethrin + PBO) (Vestergaard Frandsen Holding SA, Lausanne, Switzerland), the Olyset® Plus (permethrin + PBO) (Sumitomo Chemical Asia Pte Ltd, Health and Crop Sciences Sector, Tokyo, Japan), the Veeralin® (alpha cypermethrin + PBO) (VKA Polymers Pte Ltd, Karur, India) and the DawaPlus® (deltamethrin + PBO) (Tana Netting Co. Ltd, Dubai, U.A.E.). The benefit of the addition of PBO is only expected to manifest in areas in which mosquito populations are resistant to pyrethroids and experimental hut trials of PBO LLINs in areas of resistance have supported this prediction. Increased mosquito mortality was observed in experimental huts containing PBO LLINs compared with conventional LLINs in Ivory Coast, Benin and Burkina Faso (Corbel *et al.*, 2010; Ngousso *et al.*, 2010; Koudou *et al.*, 2011; Pennetier *et al.*, 2013) and reductions in blood feeding rates were also reported in trials in the latter two countries. All of these sites reported high levels of pyrethroid resistance.

There has been a dramatic escalation in the strength of pyrethroid resistance in southwestern Burkina Faso since earlier trials of PBO LLINs in 2007. World Health Organization cone bioassays performed in 2012 revealed that none of the conventional LLINs were effective in killing local vector populations and that the performance of the PBO LLIN PermaNet® 3.0 was also compromised in these assays (Toe *et al.*, 2014). Although the numbers of malaria deaths in Burkina Faso have fallen over the past 10 years, numbers of malaria cases have risen year on year despite countrywide LLIN distribution campaigns [National Malaria Control Programme (NMCP), personal communication; K.H.Toe, 2017]. In order to advise the NMCP on whether a switch to PBO LLINs may be warranted to target these highly resistant populations, an experimental hut trial was undertaken in two rice-growing areas in the southwestern region of Burkina Faso.

## Materials and methods

### Study sites

The experimental hut studies were carried out at two field stations in southwest Burkina Faso: the first is located in the Vallée du Kou 5 (VK5) near Bobo-Dioulasso (11°39' N,

04°41' W) and belongs to the Institut de Recherche en Science de la Santé (IRSS)/Centre MURAZ, and the second is located at Tengrela (10°40' N, 04°50' W) near Banfora and is maintained by the Centre National de Recherche et de Formation sur le Paludisme (CNRFP). These two sites are separated by approximately 120 km. Previous surveys revealed *Anopheles coluzzii* (formerly *Anopheles gambiae* s.s. M molecular form) to be the predominant *Anopheles* species at both sites. High levels of resistance to both DDT and pyrethroids have been reported previously at both sites (Ngufor *et al.*, 2014; Toe *et al.*, 2015).

### Characterization of mosquito populations

*Anopheles gambiae* s.l. larvae were collected in Tengrela and VK5 and reared to adults in local insectaries (mean relative humidity:  $75 \pm 10\%$ ; mean temperature:  $27 \pm 2^\circ\text{C}$ ). To assess susceptibility to pyrethroids, batches of approximately 25 non-blood-fed *An. gambiae* s.l. females aged 3–5 days were exposed to papers treated with 0.75% permethrin or 0.05% deltamethrin. The papers and susceptibility test kits were purchased from the Universiti Sains Malaysia (Penang, Malaysia). In parallel bioassays, mosquitoes were exposed to papers treated with 4% PBO [prepared by the Liverpool School of Tropical Medicine (LSTM)] for 1 h before they were transferred to tubes containing either insecticide-treated papers or insecticide-free papers and exposed for a further 1 h. Knock-down was recorded at the end of exposure and mortality was recorded 24 h later.

The bio-efficacy of the LLINs was tested using non-blood-fed mosquitoes aged 3–5 days from local larval collections, and from the Kisumu susceptible laboratory strain, using the WHO cone bioassay procedure (WHO, 2013). For each LLIN type, two unwashed nets were tested under insectary conditions. Ten mosquitoes per cone were exposed for 3 min to 30 cm × 30 cm net pieces sampled from the top, the short and the long sides of the net. During exposure, the set-up was kept at an angle of 45 degrees as recommended (Owusu & Müller, 2016). Knock-down and mortality were recorded at 60 min and 24 h after exposure, respectively. Conventional LLINs were compared with PBO LLINs using Fisher's exact test ( $2 \times 2$  contingency table, significance level of 0.05) (<http://www.graphpad.com/quickcalcs/contingency2>) (Roberts & Andre, 1994).

### Molecular analysis

DNA was extracted from mosquito legs by heating at  $90^\circ\text{C}$  for 30 min. Species were identified using the SINE200 protocol (Santolamazza *et al.*, 2008) and then screened for the voltage gated sodium channel (VGSC) 1014F and 1575Y alleles using Taqman assays (Bass *et al.*, 2007; Jones *et al.*, 2012).

Total RNA was extracted from six pools of 10 5-day-old non-blood-fed female *An. coluzzii* from larval collections from Tengrela and VK5 using the RNAqueous®-4PCR Kit for isolation of DNA-free RNA (Ambion, Inc., Austin, TX, U.S.A.) according to the manufacturer's procedures. The RNA was eluted in 50 µL of elution solution and treated with DNase. The quality and quantity of all the RNA used were assessed

using a NanoDrop ND1000 (Thermo Fisher Scientific UK Ltd, Renfrew, U.K.).

The expression profiles of five P450 genes (*CYP6M2*, *CYP6Z2*, *CYP6Z3*, *CYP6P3*, *CYP6P4*), previously found to be over-expressed in pyrethroid-resistant field populations from Burkina Faso (Toe *et al.*, 2015) and/or known to metabolize pyrethroids (Müller *et al.*, 2008; Mitchell *et al.*, 2012) were quantified using reverse-transcription quantitative polymerase chain reaction (RT-qPCR). The qPCR analysis was conducted at the LSTM and used the following mosquito populations: Ngousso, an insecticide-susceptible strain originating from Ngousso in Cameroon in 2006 and maintained in the insectary at LSTM; Tengrela specimens reared from larval collections in October–November 2014, and VK5 specimens reared from larval collections in October–November 2014. Approximately 600 ng of RNA was reverse-transcribed to first-strand cDNA using SuperScript™ III reverse transcriptase (Invitrogen, Inc., Carlsbad, CA, U.S.A.) according to the manufacturer's procedures. Samples were then purified using the Qiagen Easy Purification Kit (Qiagen Benelux BV, Venlo, the Netherlands) before proceeding to qPCR. Each of the six pool replicates were run in triplicate using 2X SYBR Brilliant III (Agilent Technologies, Inc., Palo Alto, CA, U.S.A.), forward and reverse primers (300 nM) [sequence available in Toe *et al.* (2015)] on the Mx3005P qPCR system (Agilent Technologies, Inc., Palo Alto, California) with the following cycling protocol: 95 °C for 3 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 10 s. The qPCR data were analysed using the delta Ct values method, taking into account the PCR efficiency (Pfaffl, 2001). The candidate Ct values were normalized against three house-keeping genes, encoding ribosomal protein L40 (ubiquitin) (*AGAP007927*), an elongation factor (*AGAP005128*) and the S7 ribosomal protein (*AGAP010592*). The normalized Ct values of each gene were then compared with the normalized Ct values of the susceptible Ngousso strain.

### Experimental hut trials

Each station consisted of six experimental huts built according to the West African style (WHO, 2013). The study had six arms that used, respectively, five different LLINs and one net with no insecticide treatment as a negative control (Table 1). The

nets were obtained from the manufacturers and were unpacked and kept in the shade for 24 h, but not washed prior to testing. Two of the nets, the OlysetPlus and PermaNet 3.0, contain PBO, whereas the other three LLINs contain only pyrethroids. The LLINs were holed according to WHO standard procedures (WHO, 2013). A total of six holes (4 cm × 4 cm) per net were cut, two on each of the long sides and one on each of the short sides.

Study participants (male sleepers) spent 6 nights per week under a net in an experimental hut from 20.00 hours to 05.00 hours, followed by 1 day of break. The sleepers were rotated through the six huts so that each sleeper spent 1 night per week under each net type. To complete a full Latin square rotation with all combinations of sleeper, net type and hut, the study ran over 36 days from 8 September to 22 October 2014.

Each morning at 05.00 hours, mosquitoes were collected manually by the sleepers, with supervision, from under the net, inside the hut and on the exit veranda. The collected specimens were morphologically identified to genus and, where possible, to species level (Gillies & Coetzee, 1987), grouped according to their gonotrophic stage (blood-fed, unfed or gravid), and scored as dead or alive. Live mosquitoes were transferred to paper cups, provided with 10% sugar water and kept in the insectary described above for 24 h, after which delayed mortality was recorded. All specimens were stored on silica gel for further molecular analysis.

Data analysis was performed in the open-source statistical software R Version 3.3.2 (R Development Core Team, 2011) using the libraries 'lme4' (Bates *et al.*, 2012) and 'glmADMB' (Skaug *et al.*, 2012) for generalized linear mixed models (GLMMs). Plots were then generated with the package 'ggplot2' (Wickham, 2009).

In the statistical analysis of hut trial data, in order to increase the number of replicates, give more power and increase confidence in the analysis, data collected from both sites (Tengrela and VK5) were pooled and the following four outcomes for *An. coluzzii* were compared between the LLINs and the untreated control net, as well as between the PBO and non-PBO nets from the same manufacturer: (a) deterrence (i.e. the reduction in hut entry relative to the control or non-PBO net); (b) induced exophily (i.e. the ratio of the odds of a mosquito being found in the veranda trap compared with the hut); (c) blood feeding inhibition (i.e. the ratio of the odds of blood

**Table 1.** Treatment arms and descriptions of longlasting insecticide-treated nets.

Treatment arm	Description	Manufacturer
Untreated net	Net manufactured manually using netting material from market	Local market
Olyset® Net	$8.6 \times 10^{-4}$ kg/m <sup>2</sup> of permethrin incorporated into polyethylene	Sumitomo Chemical
Olyset® Plus	$8.6 \times 10^{-4}$ kg/m <sup>2</sup> of permethrin and $4.3 \times 10^{-4}$ kg/m <sup>2</sup> of PBO incorporated into polyethylene	Sumitomo Chemical
PermaNet® 2.0	$5.5 \times 10^{-5}$ kg/m <sup>2</sup> of deltamethrin coated on polyester	Vestergaard Frandsen
PermaNet® 3.0	Combination of 2.8 g/kg of deltamethrin coated on polyester with strengthened border (side panels) and deltamethrin (4.0 g/kg) and PBO (25 g/kg)	Vestergaard Frandsen
Dawa® Plus 2.0	$8.0 \times 10^{-5}$ kg/m <sup>2</sup> of deltamethrin coated on polyester	TANA Netting

PermaNet is a registered trademark of Vestergaard Frandsen Holding SA. Olyset is a registered trademark of Sumitomo Chemical Co. Ltd. DawaPlus is a registered trademark of Tana Netting Co. Ltd. PBO, piperonyl butoxide.

fed vs. unfed mosquitoes), and (d) induced mortality [i.e. the ratio of the odds of dead vs. alive mosquitoes] (The original dataset for *An. gambiae s.l.* is supplied in Table S2.) Immediate mortality and mortality at 24 h post-collection were combined for the analysis. Deterrence was analysed as the ratio in total numbers between the treatment arms (or PBO net) vs. the control arm (or non-PBO net). The numbers of mosquitoes in the room and the veranda were combined and analysed using a GLMM with a negative binomial distribution and a log link function using the R function 'glmmadmb()' in the 'glmmADMB' package. In the model, the net type was the fixed effect term and random intercepts were introduced for the sleeper and the hut, and a random slope for the day depending on the location. For proportional outcomes of induced exophily, blood feeding inhibition and induced mortality, the negative binomial model was replaced by a GLMM with a binomial distribution and logit link function using the R function 'glmer()' in the 'lme4' package. In the models, in addition to the terms listed above, a random intercept was introduced for each observation to account for unexplained overdispersion. For statistical testing, the level of significance was set at  $\alpha = 0.05$ .

In addition to the ratios described above, averages (i.e. the modes) and 95% confidence intervals (CIs) of the crude values underlying the outcomes were computed by the same models as above but using the individual nets as the intercept. These corresponding crude values were: (a) entry rate (i.e. the number of mosquitoes entering a hut); (b) exit rate (i.e. the number of mosquitoes collected from the veranda trap); (c) blood feeding rate (i.e. the proportion of blood-fed mosquitoes), and (d) mortality rate (i.e. the proportion of dead mosquitoes at 24 h post-collection).

The study participants were recruited from the local communities and gave informed consent. Ethical approval was obtained from the Ethical Committee for Health Research of the Ministry of Health and Ministry of Research in Burkina Faso (Deliberation No. 2013-07-057, 11 July 2013). Malaria chemotherapy was not offered to study participants in line with Ministry of Health recommendations. However, medical supervision was

provided throughout the study and any malaria case was treated according to national requirements.

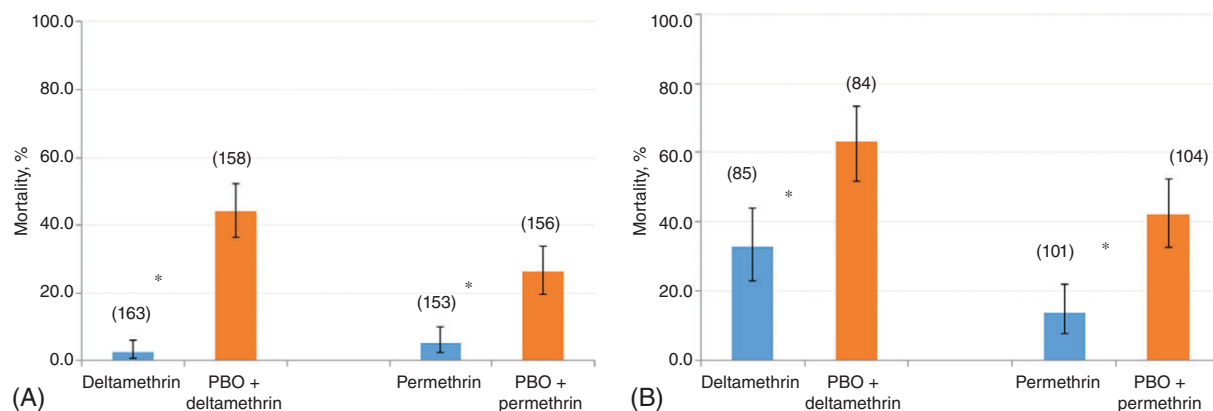
## Results

### *Pyrethroid resistance in Tengrela and VK5 and associated mechanisms*

In VK5, very low levels of mortality were observed after exposure to the discriminating dose of deltamethrin and permethrin, but pre-exposure to PBO significantly increased mortality rates from 2.5% ( $n = 163$ ) to 45% ( $n = 158$ ) and from 5% ( $n = 153$ ) to 26% ( $n = 156$ ) for deltamethrin and permethrin, respectively (Fisher's exact test,  $P < 0.0001$ ) (Fig. 1). In Tengrela, mortality rates of 34% ( $n = 85$ ) and 14% ( $n = 101$ ) were recorded for deltamethrin and permethrin, respectively. When PBO was used, mortality rates increased significantly to 63% ( $n = 84$ ) and 42% ( $n = 104$ ), respectively (Fisher's exact test,  $P < 0.0001$ ) (Fig. 1). Although there was evidence of synergism, pre-exposure to PBO did not fully restore susceptibility in either site to either pyrethroid.

*Anopheles coluzzii* was the only species of the *An. gambiae* complex identified by PCR of a subset of 80 specimens from Tengrela and VK5. High frequencies of the 1014F allele of the VGSC were recorded in Tengrela (0.819, 95% CI 0.750–0.875) and VK5 (0.885, 95% CI 0.824–0.930) with the 1575Y allele present at lower frequencies of 0.169 (95% CI 0.114–0.236) and 0.221 (95% CI 0.158–0.295), respectively. Samples were not genotyped for the 1014S allele as previous extensive surveys have not detected this allele in *An. coluzzii* from these sites (Toe *et al.*, 2015). There was no statistically significant difference in the frequency of either the 1014F or 1575Y allele between the two sites (Table 2).

Several cytochrome P450 genes previously associated with pyrethroid resistance showed elevated expression levels in the Tengrela and the VK5 *An. coluzzii* populations compared with the susceptible laboratory Ngousso strain (Fig. 2). *CYP6P3*, *CYP6M2*, *CYP6Z3*, *CYP6P4* and *CYP6Z2* were found to be



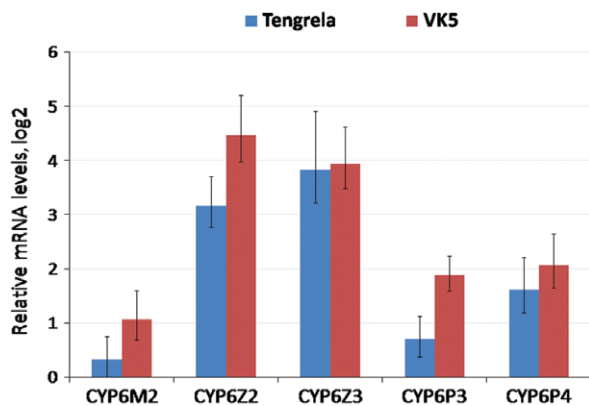
**Fig. 1.** Mortality rates (with binomial confidence intervals) after exposure to deltamethrin and permethrin in World Health Organization discriminating dose assays with or without pre-exposure to piperonyl butoxide (PBO) in (A) Vallée du Kou 5 and (B) Tengrela (October 2014). \*Significant differences in mortality:  $P < 0.0001$ . Numbers in brackets are the total numbers of mosquitoes tested. [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)].



**Table 2.** Frequencies of 1014F and 1575Y *kdr* alleles in *Anopheles coluzzii*, in Tengrela and Vallée du Kou 5 (VK5).

	1014F mutation					P-value
	Total n	LL	LF	FF	f (1014F)	
Tengrela	80	2	25	53	0.819	0.26
VK5	78	0	18	60	0.885	
	1575Y mutation					P-value
	Total n	NN	NY	YY	f (1575Y)	
Tengrela	80	57	19	4	0.169	0.39
VK5	77	49	22	6	0.221	

Chi-squared test (<http://vassarstats.net/>) for the comparison of the frequency of the 1014F and 1575Y mutations in *Anopheles coluzzii* populations from Tengrela and VK5 (October 2014). No statistical difference was observed in the frequencies of the *kdr* alleles in the two sites.



**Fig. 2.** Expression levels of candidate genes previously associated with pyrethroid resistance in Tengrela and Vallée du Kou 5. The analysis was performed using the  $2^{-\Delta\Delta C_t}$  method with data normalized against three control genes and presented as a ratio of expression levels in the Ngousso susceptible laboratory strain. Relative gene expressions were transformed to log scale before plotting to minimize large differences in gene expression. [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)].

overexpressed in both field strains compared with the susceptible laboratory colony (fold change: > 2) with the most highly overexpressed genes in both populations being *CYP6Z2* and *CYP6Z3* (Fig. 2).

#### Efficacy of LLINs under laboratory conditions

All LLINs tested showed good bio-efficacy in cone bioassays against the susceptible laboratory Kisumu strain with 60 min knock-down and 24 h mortality rates of all LLINs above the 98% and 80% WHO thresholds (WHO, 2005) (Table S1). By contrast, in tests using the field-caught mosquitoes, the mortality threshold was met only by the top panels of the PermaNet 3.0 (Fig. 3). Knock-down rates exceeded the 98% threshold for the PermaNet 3.0 in VK5 only (Table S1).

#### Experimental hut results

**Entry rate and deterrence.** In total, 12 915 specimens from four different mosquito genera were collected inside the huts (sleeping rooms and veranda traps) over the 6-week trial (Tengrela,  $n = 5808$ ; VK5,  $n = 7107$ ). Most specimens collected from the huts belonged to the *An. gambiae s.l.* species complex, accounting for 75.4% ( $n = 4379$ ) in Tengrela and 98.8% ( $n = 7020$ ) in VK5. The second most frequently collected *Anopheles* species was *An. pharoensis*, of which 49 and 19 specimens were collected in Tengrela and VK5, respectively. Other *Anopheles* mosquito species, including *An. funestus* ( $n = 3$ ), *An. nili* ( $n = 2$ ) and *An. coustani* ( $n = 3$ ), were collected in Tengrela. Additional mosquito taxa were also collected, including *Mansonia* sp. ( $n = 1330$  in Tengrela and  $n = 45$  in VK5), *Culex* sp. ( $n = 41$  in Tengrela and  $n = 23$  in VK5) and *Aedes* sp. ( $n = 1$  in Tengrela) (all: Diptera: Culicidae). Because of the low numbers of other genera, only data for *An. gambiae s.l.* were included in the analysis.

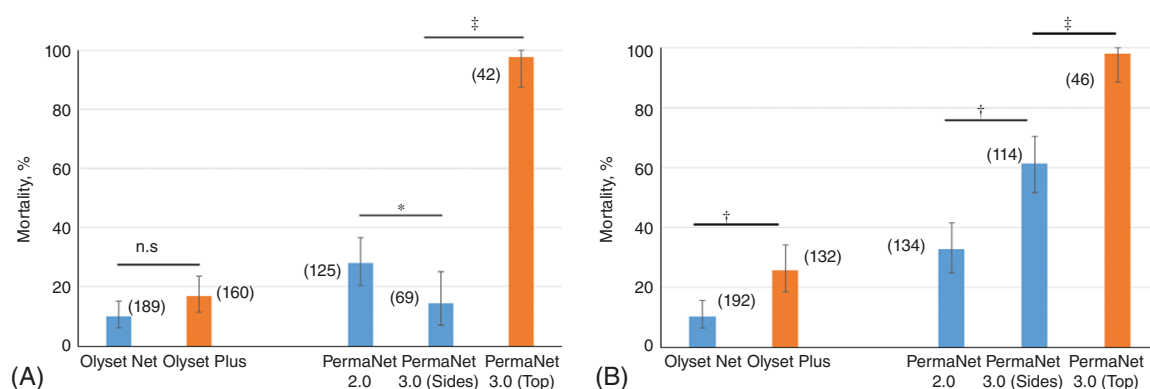
Within the total of 11 399 *An. gambiae s.l.* caught at both field sites, there was considerable variation between weeks and treatments in the numbers of mosquitoes entering the huts in both study locations (Fig. 4). The average number of mosquitoes caught per night/per hut was between 13 (PermaNet 3.0) and 20.7 (Olyset Net) mosquitoes (Table S3) and induced deterrence was found only for the Olyset Net, albeit at a low ratio of 1.31 (95% CI 1.02–1.66;  $P < 0.05$ ) (Fig. 4).

#### Exit rates and induced exophily

Exit rates refer here to the proportion of mosquitoes present in the veranda trap. As with entrance rates, exit rates varied throughout the study (Fig. 4). On average, the induced exophily rates were between 26.4% (control net) and 48.5% (Olyset Net) (Table S4). The odds of finding a mosquito in the veranda trap were significantly increased in the huts with treated nets, with the exception of the DawaPlus, although even for the DawaPlus a tendency to induce exophily was observed (Fig. 4, Table S4).

**Blood feeding rates and inhibition.** Average blood feeding rates were between 17.0% (PermaNet 3.0) and 56.4% (control net) (Table S4). With the exception of the DawaPlus, all treated nets reduced blood feeding (Fig. 4, Table S4), and the effect was most prominent with the PBO nets PermaNet 3.0 and Olyset Plus (Fig. 4, Table S4), for which the odds ratios (ORs) of blood feeding relative to control nets were 0.19 (95% CI 0.13–0.26) and 0.16 (95% CI 0.11–0.23), respectively.

**Mortality rates and induced mortality.** Average mortality rates ranged from 9.5% in the control arm to 46.1% in the PermaNet 3.0 arm (Table S4). Mortality was statistically higher in all treated net conditions than in the control huts. As with blood feeding inhibition, the PBO nets showed the largest effects in increasing mortality (Fig. 5, Table S4); the ORs for mortality relative to control nets were 5.56 (95% CI 3.92–7.89) and 8.14



**Fig. 3.** Mean mortality rates with binomial confidence intervals for mosquitoes collected from (A) Tengrela and (B) Vallée du Kou 5 after exposure to longlasting insecticidal nets (LLINs) for 3 min. Significant differences in mortality between pyrethroid-only and pyrethroid plus piperonyl butoxide-treated LLINs are indicated by \* ( $P < 0.001$ ), † ( $P < 0.0001$ ), n.s. (non-significant), ‡ ( $P < 0.0001$ ) for the comparison between the sides and top of the PermaNet 3.0. Numbers in brackets are the total numbers of mosquitoes tested. [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)].

(95% CI 5.64–11.75) for the Olyset Plus and PermaNet 3.0, respectively, compared with 2.65 (95% CI 1.87–3.75) and 3.33 (95% CI 2.35–4.74) for the Olyset Net and PermaNet 2.0, respectively.

**Impact of PBO on LLIN efficacy in pyrethroid-resistant mosquitoes.** The performance of nets containing PBO as compared with pyrethroid-only nets from the same manufacturer is shown in Fig. 5. The PermaNet 3.0 deterred more mosquitoes than the PermaNet 2.0 (OR = 0.67,  $P < 0.01$ ) (Fig. 5, Table S5), but there was no significant difference between the Olyset Plus and Olyset Net ( $P = 0.412$ ). The Olyset Net induced more exophily than the Olyset Plus (OR = 0.76,  $P < 0.05$ ), but there was no significant difference in exophily between the PermaNet 2.0 and 3.0 ( $P = 0.727$ ) (Table S5). The PBO nets from both manufacturers considerably reduced blood feeding compared with non-PBO nets with ORs of 0.34 ( $P < 0.001$ ) and 0.55 ( $P < 0.01$ ) for the PermaNet 3.0 and Olyset Plus, respectively (Fig. 5, Table S4). In addition, the PBO nets killed significantly more *An. gambiae* s.l. than the non-PBO nets. The ORs for mortality were 2.45 ( $P < 0.001$ ) for the PermaNet 3.0 and 2.1 ( $P < 0.001$ ) for the Olyset Plus (Fig. 5, Table S5).

## Discussion

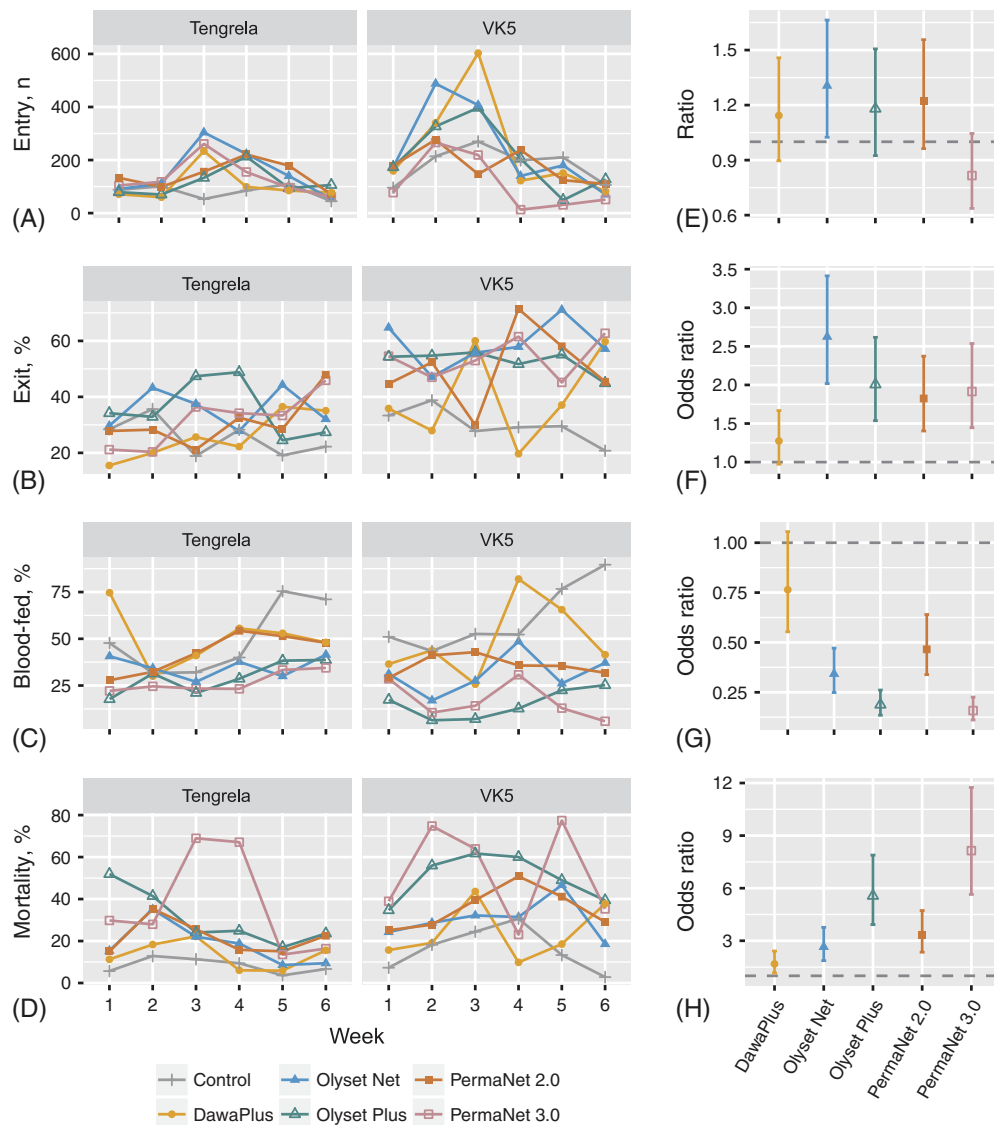
### Pyrethroid resistance in southwest Burkina Faso

Very low mortality rates were obtained for both permethrin and deltamethrin in the two study sites following an hour of exposure to WHO diagnostic doses. Southwestern Burkina Faso is known as a hotspot of pyrethroid resistance (Dabiré *et al.*, 2012; Namountougou *et al.*, 2012). Rapid changes in the prevalence of pyrethroid resistance have been observed since the first national LLIN distribution programme in 2010: in 2009, mosquito mortality following deltamethrin exposure was 25% (Dabiré *et al.*, 2012), in VK has since fallen to just 2.5%. The frequency of the 1014F *kdr* mutation also

increased from 0.28 in 2006 (Dabiré *et al.*, 2009) to 0.88 in the current study. Similar increases in the prevalence of pyrethroid resistance have been witnessed in Tengrela. Mortality rates of 93% and 46% for deltamethrin and permethrin, respectively, were recorded in 2011 (Namountougou *et al.*, 2012; K. H. Toe, unpublished data 2011), but these mortality rates had reduced to 33% and 13% in 2014, the year of the current study (Toe *et al.*, 2015). In the present study, pyrethroid mortality was significantly increased by pre-exposure to PBO. This, together with the qPCR data showing elevated expression of multiple P450s in both field populations compared with a susceptible laboratory strain, indicate that oxidases are an important resistance mechanism in *An. coluzzii* in southwestern Burkina Faso. It is noted that resistance was not fully restored by PBO pre-exposure, indicating that additional resistance mechanisms, such as target site resistance and possibly cuticular modifications (Toe *et al.*, 2015), may contribute to the pyrethroid resistance phenotype in these populations.

### Bio-efficacy of LLINs in cone bioassays

The low levels of mortality observed in cone bioassays in this study are similar to those reported previously in VK7, a neighbouring village to VK5, in the Vallée du Kou rice-growing region. However, whereas the current study found that exposure to the tops of the PermaNet 3.0 nets resulted in mortality levels exceeding the WHO threshold of 80%, equivalent bioassays conducted in the VK7 population in 2012 showed mortality of only 43%. Cone bioassays are not a reliable method of comparing the performance of LLINs containing different pyrethroids because of the inherent differences in excito-repellency within this class, which can affect exposure time (Siegert *et al.*, 2009). In particular, cone tests may underestimate the performance of permethrin LLINs; this is indicated by comparison of the cone bioassay results in Fig. 3, in which the Olyset Net was found to induce considerably lower mortality than the PermaNet 2.0, although experimental hut results showed similar levels of mortality in huts with these two net types (Table S3).



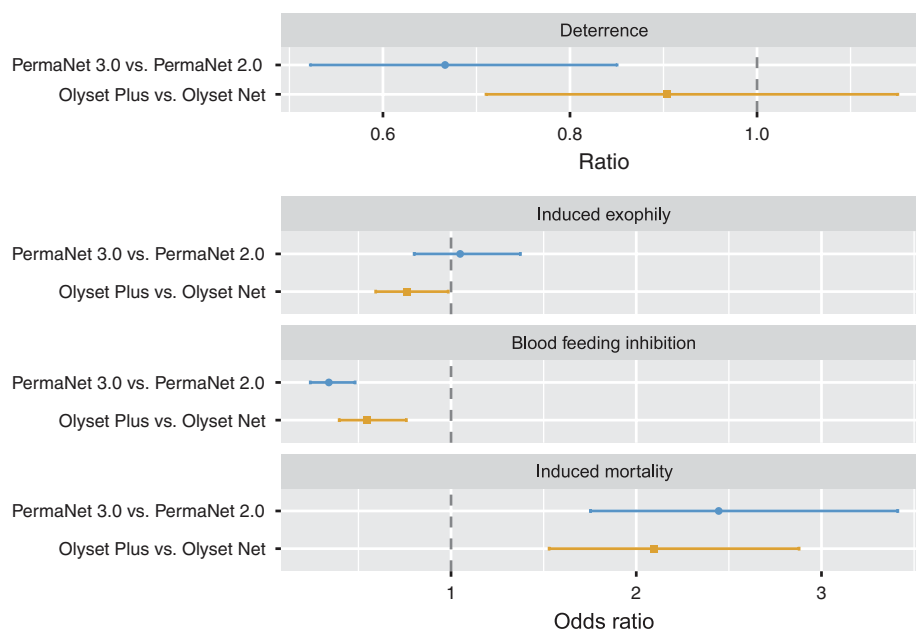
**Fig. 4.** Primary outcomes measured in the experimental hut trials in Tengrela and Vallée du Kou 5 (VK5). (A–D) Crude data for the measured outcomes. (A) Entry, number of mosquitoes per night entering a hut. (B) Exit, proportion of mosquitoes collected from the veranda trap. (C) Blood feeding, proportion of mosquitoes found blood-fed. (D) Mortality, proportion of mosquitoes dead at 24 h post-collection. (E–H) These outcomes measured relative to the control (i.e. untreated mosquito net) for both sites combined. (E) Deterrence, the ratio of mosquitoes entering a hut relative to the control hut. (F) Exophily, the odds ratio (OR) for a mosquito being found in the veranda trap. (G) Blood feeding inhibition, the OR for a mosquito being blood-fed. (H) Mortality, the OR for a mosquito being dead in the morning (immediate mortality) or after being caught alive and held for 24 h (delayed mortality). Symbols show the average and whiskers show the limits of the 95% confidence intervals around the average. The dashed line shows the ratio or OR of 1, indicating no association of the outcome with the treatment. The data used to produce the plot together with the *P*-values are provided in Table S3. [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)].

Nevertheless, the results of these cone bioassays provide further evidence that resistance can be at least partially ameliorated by exposure to PBO.

#### Performance of PBO LLINs in experimental hut studies

The enhanced performance of PBO-containing LLINs over conventional LLINs was further supported by the experimental

hut results. Both blood feeding inhibition and mortality rates were significantly higher in huts containing PBO LLINs than in those containing conventional LLINs from the same manufacturers. An improved performance of the PermaNet 3.0, which contains both higher concentrations of deltamethrin compared with the PermaNet 2.0, plus PBO on the roof of the net, has also been reported in pyrethroid-resistant populations of malaria vectors in Ivory Coast (Koudou *et al.*, 2011), Benin (N'Guessan *et al.*, 2010) and Burkina Faso (Corbel *et al.*, 2010).



**Fig. 5.** Outcome measures comparing piperonyl butoxide (PBO)-treated and non-PBO-treated nets from the same manufacturers. Deterrence, the ratio of mosquitoes entering a hut relative to the control hut. Exophily, the odds ratio (OR) for a mosquito being found in the veranda trap. Blood feeding inhibition, the OR for a mosquito being blood-fed. Mortality, the OR for a mosquito being dead in the morning (immediate mortality) or after being caught alive and held for 24 h (delayed mortality). [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)].

The magnitude of this effect differs among studies, with previous studies reporting increases in mortality rates of 1.3–1.8-fold, whereas the current study reports an OR of 2.45, which corresponds to a 1.78-fold increase. Only one experimental hut study comparing the Olyset Plus with the Olyset Net has been published to date (Pennetier *et al.*, 2013). This study, conducted in Benin in 2013, reported mortality rates 1.9-fold higher in the PBO arm than in the conventional LLIN arm (Pennetier *et al.*, 2013), which is in line with the findings of the current study, which found a 1.89-fold (OR 2.1) elevation in mortality rates in the Olyset Plus arm.

In addition to increased mortality rates, the current study, plus two of the previous experimental hut studies, reported significantly higher rates of blood feeding inhibition for PBO vs. conventional LLINs (Corbel *et al.*, 2010; N'Guessan *et al.*, 2010), indicating that PBO LLINs afford an enhanced level of personal protection in areas where vectors are resistant to pyrethroids. It should be noted that the current study was performed using unwashed nets only. Previous studies have found that the efficacy of PBO LLINs against resistant mosquitoes decreases substantially after the nets have been washed 20 times according to WHO protocols (Corbel *et al.*, 2010; Tungu *et al.*, 2010; Koudou *et al.*, 2011). Thus further studies on the durability of the bio-efficacy of PBO LLINs under field conditions are urgently needed.

## Conclusions

The results of these experimental hut studies with entomological endpoints suggest that substituting conventional LLINs

with PBO LLINs in areas where there is a high prevalence of pyrethroid resistance in local vectors may be an effective strategy to maintain the efficacy of malaria vector control. The public health benefit of this would depend on a wide range of factors, including the level of malaria endemicity, LLIN coverage rates and the predominant mosquito vectors present, but a recent modelling exercise predicts that in some settings, a switch to PBO LLINs could avert up to 0.5 clinical cases per person per year (Churcher *et al.*, 2016). These predictions from models are now being evaluated in large-scale field trials. A recent study in Tanzania reported a 33% protective efficacy of PBO LLINs over conventional LLINs after 2 years of use (Protopopoff *et al.*, 2018). A larger trial, involving the distribution of over 10.7 million nets in Uganda, is evaluating whether PBO nets reduce malaria prevalence under programmatic conditions (<https://www.againstmalaria.com>, <https://www.pmi.gov>).

In light of the results from experimental hut studies, including the current study, and after reviewing data from the first clinical trial of a PBO LLIN, the WHO recently made a policy recommendation that national malaria control programmes should consider deployment of PBO LLINs in areas with pyrethroid-resistant vectors (WHO, 2017). If deployed at scale, PBO LLINs may play an important role in reducing the immediate threat of pyrethroid resistance to malaria control.

## Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.



**Table S1.** Knock-down at 60 min and 24 h mortality rate with 95% binomial confidence intervals of the longlasting insecticidal nets against Kisumu, Tengrela and Vallée du Kou 5 colonies. Data for the PermaNet 3.0 refer to combined results from the top and sides of the net.

**Table S2.** Original dataset with data for *Anopheles gambiae* s.l.

**Table S3.** Outcomes measured in the experimental hut trial when pooling data from both study sites.

**Table S4.** Comparisons of longlasting insecticidal nets vs. the untreated control net for each brand. Data from both sites were combined for this analysis.

**Table S5.** Comparison of piperonyl butoxide (PBO)-treated longlasting insecticidal nets (LLINs) with the respective non-PBO-treated LLIN.

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